American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Immunohistochemical Testing of Estrogen and Progesterone Receptors in Breast Cancer (Unabridged Version)

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• *Purpose.*—To develop a guideline to improve the accuracy of immunohistochemical (IHC) estrogen receptor (ER) and progesterone receptor (PgR) testing in breast cancer and the utility of these receptors as predictive markers.

***Methods.*—The American Society of Clinical Oncology and the College of American Pathologists convened an international Expert Panel that conducted a systematic review and evaluation of the literature in partnership with Cancer Care Ontario and developed recommendations for optimal IHC ER/PgR testing performance.**

***Results.*—Up to 20% of current IHC determinations of ER and PgR testing worldwide may be inaccurate (false negative or false positive). Most of the issues with testing have occurred because of variation in pre-analytic variables, thresholds for positivity, and interpretation criteria.**

***Recommendations.*—The Panel recommends that ER and PgR status be determined on all invasive breast cancers and breast cancer recurrences. A testing algorithm that relies on accurate, reproducible assay performance is proposed. Elements to reliably reduce assay variation are specified. It is recommended that ER and PgR assays be considered positive if there are at least 1% positive tumor nuclei in the sample on testing in the presence of expected reactivity of internal (normal epithelial elements) and external controls. The absence of benefit from endocrine therapy for women with ER-negative invasive breast cancers has been confirmed in large overviews of randomized clinical trials.**

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ESTROGEN RECEPTOR PHYSIOLOGY AND MEASUREMENT

The estrogen receptor (ER) is the paradigm tumor marker for management of patients with cancer. For more than three decades, ER has been the most important biomarker measured for the management of breast cancer, largely because of the substantial benefit that endocrine therapy provides for ER-positive but not ER-negative tumors in women of all ages.[1,2] The clinical significance of ER has rendered the assessment of ER status of primary invasive

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breast cancer mandatory.[3–5] The accuracy of the method used determines the appropriate application of treatment and leads to significant improvements in survival. During the more than 30 years of analysis of ER, several methods with differing biochemical principles, analytic sensitivity, and analytic precision have been used.[6–8]

Estrogen is produced via two different organ systems. In premenopausal women, the vast amount of estrogen (estradiol-17β and estrone) is produced by the ovaries in response to the pituitary-derived luteinizing and follicle-stimulating hormones. In postmenopausal women, the ovaries produce few or no estrogenic compounds. Rather, precursors of estrogen (testosterone and androstenedione) are produced by the adrenal gland and converted in peripheral tissue to estradiol and estrone via aromatization as a result of enzymatic products of the gene *CYP19*. Estradiol levels in premenopausal women range from 15 to 200 pg/mL (depending on the menstrual cycle), whereas levels in postmenopausal women are generally less than 10 to 20 pg/mL (36.71 to 73.42 pmol/L).[9,10]

Estrogen signaling occurs via the standard classical steroid receptor mechanism.[11] Estrogen freely diffuses through the cellular membrane and binds monomeric ER protein, which seems to be almost exclusively nuclear even in the absence of ligand. The binding of estradiol leads to a conformational change in ER and induces receptor dimerization. The ligand/receptor complex then binds directly or indirectly to estrogen response elements in the promoter regions of estrogen-responsive genes enhancing transcription. The precise cellular response depends on tissue-specific nuclear ER co-regulatory proteins, designated co-activators, and co-repressors. There are two separate but highly homologous ER isoforms, ERα and ERβ, encoded by two separate genes, *ESR1* and *ESR2*, respectively. The precise cell-specific physiologic and pathophysiologic roles of ERβ are unclear at present. There are no data supporting any clinical role of evaluation of ERβ at present, and therefore, the term ER refers to the product of *ESR1* (ERα) throughout this guideline document.

Measurement and characterization of ER protein was first reported by Jensen et al[12] in the mid-1960s. The first indications of clinical importance of ER as a prognostic or predictive factor were reported by McGuire and others in the early 1970s.[13,14] Until about 1990, ER protein was quantified using a variety of ligand-binding assays (LBAs) that depended on homogenization of fresh-frozen tumor followed by centrifugation to provide a crude cytosol. Incubation of portions of cytosol with increasing quantities of radioactive estradiol-17β in a competitive fashion together with known amounts of nonradioactive estrogen mimics was followed by separation of the bound and unbound estrogen to allow the concentration of high-affinity estrogen binding sites (ie, ER) to be quantified, using Scatchard plots and standard curves of known amounts of the protein. Early studies used density gradient centrifugation to separate bound from free estradiol, but subsequent studies most commonly used dextran-coated charcoal (DCC) to separate the receptor-bound estrogen, such that the terms LBA assay and DCC assay became synonymous in most minds.[6]

The availability of monoclonal antibodies to ER in the 1980s had a profound and long-lasting impact on the methodologies for ER assay. Initially, quantification was by enzyme immunoassay that still required preparation of cytosols from homogenates of fresh tissue but was more precise and far less laborious than the DCC assay. The results from the new structurally specific assay were highly correlated with the old functionally specific assay,[15] and cutoff values approved by the US Food and Drug Administration (FDA) for enzyme immunoassay (ER positivity ≥ 15 fmol/mg cytosol protein) were mostly based on correlative values with LBA (ER positivity ≥ 10 fmol/mg cytosol protein). In addition, use of optimal cutting temperature embedding compound with frozen breast tumors did not alter either ER or PgR results. Immunohistochemical (IHC) application of the hormone receptor–specific antibodies was initially successful only on sections of frozen tissue, but antigen retrieval methods developed in the early 1990s and new antibodies allowed application to routinely formalin-fixed and paraffin-embedded material. The ease of this approach to analysis, the availability of inexpensive reagents that are applicable to routine pathology specimens, and importantly, the ability to evaluate small cancers and ensure that only invasive tumor cells are assessed have led to IHC becoming the near universal choice for ER and PgR assay determinations since the early 1990s.

However, much of the data that underpin our knowledge of the relationship between the presence of significant amounts of ER and response to endocrine therapy were derived using an LBA, and positive/negative cutoff values were developed by reference to those results.[1,2,14,16] Some of the newer analytic IHC methods have been subject to retrospective comparisons with established methods such as LBA, and there are some published reports that IHC may be more predictive in identifying patients who will derive benefit from endocrine therapy (Table 1).

The IHC assays identify ER as a nuclear protein, and cell fractionation experiments largely support this observation. Signaling through the mechanism described earlier is designated the genomic signaling pathway.[11] However, recent laboratory studies using cultured human breast cancer cell lines have demonstrated that ER may signal through a separate, nongenomic pathway. In this case, ER has been found to be associated with the breast cancer cell membrane, and its effect is mediated through a cascade of tyrosine kinases and phosphatases, ultimately resulting in gene activation through nuclear transcription factor modulation.[23] In classically estrogen-dependent cell lines, such as MCF-7, this mechanism may result in acquired endocrine resistance, but to date, there has been no demonstration of such localization in clinical samples, and the importance of this mechanism in human breast cancer and its treatment is unknown.

Progesterone receptor (PgR) is also frequently measured for the management of breast cancer.[3] Initial reports, which have been validated through the years, suggested that patients with ER-positive/PgR-negative breast cancers have a worse prognosis than patients who have PgR-positive tumors.[24] However, suggestions that PgR would serve as an important predictive factor for benefit from antiestrogen therapies have not been borne out in subsequent studies.[25–27] The latter hypothesis was based on the observation that the gene encoding for PgR is estrogen dependent and, thus, PgR expression might serve as an indication of an intact estrogen-ER-estrogen response pathway.[28] Like ER, PgR was initially measured by forms of LBA but is now measured almost exclusively by IHC. Also like ER, PgR protein exists as two receptor

Table 1. ER Expression by Original LBA and Retrospective IHC Versus Benefit from Endocrine Therapy (Selected Trials)							
Reference	No. of Patients (eligibility)	Intervention (outcome)	Original Assay (cutoff)	Retrospective Assay (cutoff)	Assay Concordance	Outcome According to Biomarker	Comments
McCarty et al[17]	Pop A, n = 62 (early stage); Pop B, n = 72 (early stage); Pop C, n = 23 (MBC)	Endocrine Rx (Pop C)	LBA (≥ 20 fmol/mg)	H222 Sp γ Pop (score 75)	Pop A = specificity, 89% and sensitivity, 95%; Pop B = specificity, 94% and sensitivity, 88%	Objective clinical response: specificity, 89%; sensitivity, 93%	Among the original reports describing IHC correlation with LBA and with response to endocrine Rx
Barnes et al[18]	170 patients; 74% ER positive by LBA	First-line TAM in MBC (51% response rate)	LBA; 74% ER positive (≥ 20 fmol/mg); response rate, 58%	IHC with ER 1D5 antibody; 31% to 69% ER positive (various IHC scoring methods); response rate, 64% to 69%	137 (81%) of 170	Responses in 72% of ER/PgR positive and 61% of ER positive/PgR negative; IHC superior for predicting duration of response	All 8 IHC scoring methods useful
Harvey et al[19]	1,982 patients	26% received endocrine Rx and 13% received combined chemo-endocrine Rx	LBA (positive if ≥ 3 fmol/mg)	IHC with 6F11 (Allred score > 2 or 1% to 10% weakly positive cells)	71% of all tumors were ER positive by IHC (86% concordance with LBA)	Multivariate analysis of patients tested by LBA showed ER status determined by IHC better than by LBA at predicting better DFS	This study was based on samples prepared in an unconventional manner (see text for details)
Elledge et al[20]	205 patients with blocks (original n = 349, all ER positive by LBA)	SWOG 8228, TAM 10 mg twice a day (n = 56) or 10 mg/m^2 twice a day (n = 149)	LBA (positive if ≥ 3 fmol/mg)	IHC with ER-6F11 antibody (Allred score)	185 (90%) of 205 were IHC positive	Overall response rate of 56% if LBA positive and 60% if IHC positive; significant correlation between IHC ER and response (ER negative, 25%; intermediate, 46%; and high, 66%) and time to Rx failure (ER negative, 5 months; intermediate, 4 months; and high, 10 months)	In low ER by LBA (< 50 fmol/mg), response rate of 25% if IHC negative and 63% if IHC high

isoforms (in this case, called A and B), but these forms are the products of the same gene. These isoforms of PgR (A is a slightly truncated form of B) bind with one another to create homo- and heterodimers. Although this is an active area of research, there has been little work on the relative significance of these forms in clinical tissue specimens.

WHY MEASURE ER AND PgR?

Since its discovery, ER has been an appealing marker to manage patients with breast cancer. Endocrine treatment for breast cancer was first reported in the late 1890s by Beatson,[29] when he observed what would now be called responses in a few young women with apparent locally advanced breast cancer after he performed surgical oophorectomy. Beatson presumed that he was interrupting neural connections between the ovaries and the breast, although he was actually removing the ligand estrogen from the patient's circulation, which diminished estrogen availability to its protein receptor (ER). Subsequent clinical trials have demonstrated that some form of

Continued							
Reference	No. of Patients (eligibility)	Intervention (outcome)	Original Assay (cutoff)	Retrospective Assay (cutoff)	Assay Concordance	Outcome According to Biomarker	Comments
Thomson et al[21]	332 patients (premenopausal patients with stage II disease); 81% had tumor assayed for ER by LBA	Adjuvant OA *v* CMF chemotherapy	LBA originally done in 270 patients or 81% (negative if < 20 fmol/mg with 2 categories, or negative if 0–4 fmol/mg with 4 categories)	IHC done in 236 patients or (71%; quick score)	Spearman's rank correlation coefficient, 0.55	Significant interaction between IHC quick score and Rx with OA more beneficial for patients with positive quick score, whereas patients with quick score of 0 had significantly higher risk of death with OA	Original trial = better outcome with OA if ER > 20 fmol/mg *v* with CMF if ER < 20 fmol/mg
Regan et al[22]	571 patients [premenopausal (IBCSG trial VIII)] and 976 patients [postmenopausal with node-negative disease (IBCSG trials VIII and IX)]	IBCSG trial VIII (none, CMF, goserelin, or CMF → goserelin); IBCSG trial IX (TAM or CMF → TAM)	55% patients had LBA (positive if ≥ 20 fmol/mg) and 45% had ELISA	IHC with 1D5 antibody (present if > 0% stained cells and positive if ≥ 10% stained cells)	Concordance of 88% (κ = 0.66) in postmenopausal patients	HR similar for association between DFS and ER status (all patients) or PgR status (postmenopausal patients) as determined by the various methods	

Abbreviations: ER, estrogen receptor; LBA, ligand-binding assay; IHC, immunohistochemistry; Pop, population; MBC, metastatic breast cancer; Rx, therapy; TAM, tamoxifen; PgR, progesterone receptor; DFS, disease-free survival; SWOG, Southwest Oncology Group; CMF, cyclophosphamide, methotrexate, and fluorouracil; OA, ovarian ablation; IBCSG, International Breast Cancer Study Group; ELISA, enzyme-linked immunosorbent assay.

endocrine treatment substantially reduces morbidity and mortality in patients with breast cancer and is also an effective chemopreventive strategy.[30–33] There are several endocrine strategies for breast cancer treatment, including surgical ablation of estrogen-producing organs, such as the ovaries and the adrenal glands, or of the pituitary gland. More recently, medical ablation of estrogen production has been successfully achieved with the use of antagonists or refractory agonists of the gonadotropin-releasing factor in premenopausal women or by selective inhibition of aromatase activity in postmenopausal women. Estradiol was found to be an effective agent in premenopausal women if delivered in pharmacologic doses[34,35] and at doses as low as 6 mg orally daily.[36]

The development of specific pharmacologic agents that bind ER has mostly replaced other therapies. Indeed, the use of tamoxifen is one of the most important advances in all of oncology. Tamoxifen was initially developed as an antiestrogenic contraceptive but was quickly found to be an effective treatment for breast cancer.[37] Subsequent studies have found that tamoxifen has enigmatic, tissue-specific antagonistic and agonistic effects by virtue of binding to ER, inducing dimerization and nuclear translocation but mediating different co-activator and co-repressor binding than the native ligand estrogen. Tamoxifen is now designated as a selective ER modulator (SERM) rather than as an ''antiestrogen.'' Regardless, tamoxifen has resulted in remarkable benefits for women with breast cancer in all settings. In contrast, the selective estrogen receptor downregulator fulvestrant binds to ER and downregulates it, thus functioning as a pure antiestrogen. The benefits of fulvestrant have been demonstrated in the metastatic setting, and clinical trials in the adjuvant setting are ongoing.

The aromatase inhibitors (AIs) are a class of agents that block conversion of adrenally produced precursor compounds to estrogenic molecules, particularly estradiol and estrone, in peripheral tissues and within breast cancers themselves. There are three commercially available AIs—anastrozole, letrozole, and exemestane. Prospective randomized trials have demonstrated that the AIs are slightly more effective than tamoxifen in both the metastatic and adjuvant settings,[38,39] and they are now widely used in both settings.

Given these enormous benefits of endocrine therapy, one must question the wisdom of withholding it from any patient with breast cancer, and in fact, it has been recommended that a trial of endocrine therapy for any woman with metastatic breast cancer might be appropriate.[40] However, the absence of benefit from endocrine treatments for women with ER-negative invasive breast cancers has been confirmed in large overviews of randomized clinical trials.[30]

Although safer and better tolerated than chemotherapy, endocrine therapies may produce bothersome adverse effects and occasionally life-threatening toxicities such as induction of thromboembolic events and uterine cancers. Furthermore, some of these agents are quite expensive,

making them less affordable in developing health care systems.

The implication of these findings extends beyond higher income countries. To the surprise of many, cancer is second only to cardiovascular deaths in terms of absolute numbers of deaths in lower and lower-middle income countries, ahead of many communicable diseases.[41] In fact, breast cancer is the most common cancer among women worldwide, and many lower and lower-middle income countries in Africa and Asia have mortality/incidence ratios that exceed those of countries in North America, Europe, or Australia.[42] Although these mortality/incidence ratios suggest that the incidence of ER-negative breast cancer would be elevated, several available regional data sets in Africa and Southeast Asia suggest that most breast cancers in those countries are also ER positive (Table 2).[43–47] These data highlight the potential worldwide public health impact that could result from greater availability of accurate determination of ER, especially if combined with access to potentially more affordable endocrine therapy.

Although guidelines have called for routine measurement of PgR along with ER, the precise role of PgR in patient management has not been strongly established. Certainly, endocrine therapy should not be withheld from a patient with an ER-rich, PgR-poor breast cancer, and the benefit of endocrine therapy in ER-negative, PgR-positive breast cancer, which is an unusual category of tumors, is controversial.[30] However, there is evidence that the small proportion of patients with ER-negative/PgR-positive disease may respond to endocrine therapy.[14,48] This, together with prognostic information, suggests sufficient clinical value for the routine measurement of PgR. Therefore, access to accurate hormone receptor testing is critical. Given the benefits of reducing the risk of recurrence and death in the adjuvant setting and the extraordinary palliation in the metastatic setting, false-negative results could adversely affect the clinical management of a patient with breast cancer. At the same time, given the potential toxicity and cost of endocrine treatments, false-positive tests will result in inappropriate overtreatment.

Similarly, human epidermal growth factor receptor 2 (HER2) serves as an important marker for benefit from therapies targeting this receptor tyrosine kinase, such as trastuzumab and lapatinib. In 2007, a joint committee convened by the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) addressed the technical and analytic issues related to accurate HER2 testing[49,50] and issued a set of guidelines that have had a positive impact on evaluation and treatment of women with breast cancer. On the basis of this experience, ASCO and CAP have partnered again to address ER and PgR hormone receptor protein testing and to propose new guidelines that we maintain will serve to improve care of women with breast cancer worldwide. In this current guideline, the ASCO/CAP Hormone Receptor Testing in Breast Cancer Panel has limited its recommendations to IHC testing for ER and PgR because IHC remains the standard assay platform for measurement of these hormone receptor proteins in 2010. Future versions of the guideline may include other methods and platforms such as functional and genomic assays.

IS THERE A PROBLEM?

ER testing by LBA was subjected to stringent quality assurance measures and surveys conducted by cooperative clinical trial groups (eg, the National Surgical Adjuvant Breast and Bowel Project [NSABP], Southwest Oncology Group [SWOG], Eastern Cooperative Oncology Group [ECOG], North Central Cancer Treatment Group [NCCTG], Cancer and Leukemia Group B [CALGB], and Southeastern Cancer Study Group)[7,8,51,52] and entered standard of care for evaluation and treatment of breast cancer patients in the mid-1970s. LBA testing was almost entirely replaced by IHC testing in the early to mid-1990s based on assumptions that the assay was accurate according to several concordance studies between IHC and LBA. However, there have been few, if any, systematic recommendations or guidelines regarding optimization or reproducibility of IHC testing procedures, and there has been no standardized proficiency testing in the United States for ER and PgR. In the United Kingdom, there has been an external quality assurance program, United Kingdom National External Quality Assessment Service (UK NEQAS), for hormonal receptors since 1994, and in 1998, a survey evaluated the frequency of hormone receptor–positive cancers in 30% of its participating laboratories involving more than 7,000 patients.[53,54] This publication highlighted significant variation in ER and PgR positivity rates. Similar studies identified a significant number of laboratories producing false-negative results on specimens that exhibited low receptor positivity. Proficiency testing for ER, PgR, and HER2 was developed by the Royal College of Pathologists of Australasia Quality Assurance Program in 2001, and participation is mandatory in Australia and New Zealand. Data on more than 8,000 patients from two audits were reported in 2007.[55] These results also indicated significant variation across laboratories in the reporting of ER, PgR, and HER2 status. There is no current national or international guideline for ER and PgR testing that could potentially decrease this testing variation.

Recent experience and publications have addressed ER testing reliability in situations where there is a lack of a national guideline. Canada has a provincial-based health care system in which each province develops its own method of assuring health care quality measures. This results in significant variation in policies and practices among the provinces. A recent inquiry into ER testing practices in Newfoundland revealed that approximately one third of 1,023 ER tests performed on patients in that Canadian province between 1997 and 2005 were scored falsely negative when retested in a central laboratory in Ontario. More than 100 of these patients have since died, and a class action law suit has commenced, claiming negligence in ER testing and thus harm as a result of failure to provide adjuvant tamoxifen. The inquiry revealed that this testing debacle occurred because of turnover and lack of relevant training of pathologists and technologists doing the testing, lack of appropriate quality assurance methods, inadequate quality control policies and practices, and poor communication and teamwork among the health care professionals. In fact, all ER tumors that were identified as falsely negative were found to have one or more of the following three characteristics: poor fixation, negative internal control (when normal duct epithelium was present, it was negative), and absent

Table 2. Prevalence of ER-Positive Breast Cancer in Lower/Lower-Middle Income Countries							
Country	Reference	Period	No. of Patients	Age	ER and/or PgR Positive	HER2 Positive	Comments
Philippines	Uy et al[43]	2003–2005 and 2006	1,000	52% > age 50 years	2003–2005 (n = 638): 59.4% (< age 50, 59.4%; ≥ age 50, 54.5%); 2006 (n = 362): 68.9% (< age 50, 74%; ≥ age 50, 62.7%)	—	Improvement occurred after standardization of procedures regarding specimen fixation (especially for surgical specimens where lower frequency of ER-positive results observed)
Vietnam/China	Nichols et al[44]	1993–1999	682	Median, 41 years (range, 24–57 years)	61%	35%	All premenopausal
Malaysia	Tan and Yip (R.R. Love, personal communication, December 2008)[45]	2005–2007	996	Mean, 53.3 years (87% > age 40 years)	61%	30%	Triple negative, 18%
Nigeria	Adebamowo et al[46]	2004–2006	192	Mean, 49.5 years (range, 27–74 years)	65.1% ER positive, 54.7% PgR positive	20.1%	
Bangladesh	Mostafa et al (R.R. Love, personal communication, December 2008)[47]	2003–2008	1,339	Mean, 45.6 years	66.7% ER positive, 70% PgR positive	30.7% (450 tested)	

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor.

internal controls (no internal control to evaluate).[56] A similar problem has recently been publicized in Quebec, where health care quality policies for anatomic pathology laboratory testing are defined on a hospital-by-hospital basis.[57,58]

Similar assay testing issues have been highlighted in other parts of the world. Older reports of breast cancer from sites in Asia have reported that breast cancer in Asian women is mostly ER negative. However, the percentage of ER-positive tumors in a series of 1,000 patients from the Philippines was 68.9% after changes in fixation were instituted.[43] In other series from Bangladesh (R.R. Love, personal communication, December 2008),[47] Vietnam and China,[45,59] Malaysia (R.R. Love, personal communication, December 2008),[45] and Nigeria,[46] more than two thirds of patients had exhibited ER- and PgR-positive disease after implementation of standardized practices for procurement and formalin fixation of breast cancer specimens for hormone receptor assays.

Recently, reports from large multinational cooperative groups addressed concordance between ER testing at the primary institutional site of patient enrollment and centrally by high-volume, academic laboratories.[22,60,61] The International Breast Cancer Study Group (IBCSG) conducted a series of studies comparing chemoendocrine therapy to endocrine therapy alone in years before the establishment of IHC testing. Most, if not all, of the ER testing performed at the primary institutions used some form of extraction assay, either an LBA or an enzyme-linked immunoassay (ELISA), and these results were compared with results obtained after the primary tumor blocks were collected and reanalyzed in a single central laboratory using IHC. Specimens were considered positive for ER by the extraction assays if the value obtained was ≥ 10 units/mg cytosol protein (a unit depended on which of the two types of assays were performed; Table 3).[22] It is difficult to determine which is the gold standard in this study because LBA or ELISA might be considered the established assay, with the newer IHC being considered the investigational assay, or alternatively, one might consider central testing with one assay to be more reliable than individual sites. Regardless, the discordant ER results between institutional and central results were 16% (ER positive) and 24% (ER negative) for specimens from premenopausal women, and 9% (ER positive) and 24% (ER negative) for those from postmenopausal women, with an overall concordance rate of 82% and 88% for premenopausal and postmenopausal women, respectively (Table 3).

In the Breast Cancer Intergroup trial ECOG 2197,[60] all study assays were performed by IHC using standard whole-section slides in local laboratories. After trial completion, central laboratory concordance studies were all conducted using a single assay performed on specimens placed into a tissue microarray. For the original local testing, the designation of positive was left to the discretion of the primary institutional criteria, whereas in the central facility, ER was considered positive if Allred scoring criteria were ≥ 3. In this study, 11% of local ER-negative tests were scored positive on central testing, for an overall concordance rate for ER of approximately 90% (Table 4).

Table 3. Concordance Between Primary Institution and Central Testing for ER in IBCSG

ER Testing at Primary Institution (extraction assay, positive >10)	ER Central Testing (IHC, positive > 10%) Positive No.	Positive %	Negative No.	Negative %	Total No.	Overall Concordance (%)* Rate	Overall 95% CI	Positive Concordance (%)† Rate	Positive 95% CI	Negative Concordance (%)‡ Rate	Negative 95% CI
Premenopausal						81.7	78.2 to 84.8	83.8	79.9 to 87.2	75.7	67.9 to 82.3
Positive	347	84	36	24	383						
Negative	67	16	112	76	179						
Total No.	414		148		562						
Postmenopausal						87.9	85.7 to 89.9	91.5	89.2 to 93.4	76.0	69.8 to 81.5
Positive	675	91	53	24	738						
Negative	63	9	168	76	231						
Total No.	738		221		959						

NOTE. Data adapted.[22]
Abbreviations: ER, estrogen receptor; IBCSG, International Breast Cancer Study Group; IHC, immunohistochemistry.

* Overall concordance was determined using the following formula: (total No. of results that were positive in both testing locations + total No. of results that were negative in both testing locations)/total No. of results for both tests.

† Positive concordance was determined using the following formula: total No. of results that were positive in both testing locations/total No. of positive results in central testing.

‡ Negative concordance was determined using the following formula: total No. of results that were negative in both testing locations/total No. of negative results in central testing.

ER and PgR assay differences have been reported in local laboratory versus central reference laboratory testing in tumor blocks from almost 5,000 patients from countries worldwide (except United States and China) enrolled in the ongoing Adjuvant Lapatinib and/or Trastuzumab Treatment Optimization (ALTTO) trial between June 2007 and November 2008 and submitted for pre–random assignment confirmation of HER2 status at the European Institute of Oncology. Pre–random assignment central review of ER and PgR is also being performed; thus far, 4.3% of tumors that tested ER positive in local laboratories were found to be negative (false positive) on central review (Table 5). More than 20% of tumors that tested locally as ER negative were shown to exhibit at least some expression of ER (false negative) on central review.[62]

Viale et al[61] examined the prognostic and predictive value of both local and central hormone receptor expression on disease-free survival for 3,650 patients who had been assigned to the single-agent arms in the Breast International Group (BIG) 1–98 adjuvant trial testing evaluating letrozole and tamoxifen. Using criteria of ER and/or PgR ≥ 10% of positive cells, central review confirmed 97% of tumors as hormone receptor positive. However, of 105 tumors that tested as ER negative locally, 73 were found to have ≥ 10% positive cells, and eight had 1% to 9% positive cells, whereas of 6,100 tumors that tested as ER positive locally, 66 were found to have no staining, and 54 had 1% to 9% positive cells (Table 6).[61]

These data suggest ranges of inconsistent ER and PgR results in the setting of international clinical trials. However, all of the cited trials suffer from differences in methods and thresholds between central and local institutional testing, which make valid conclusions about the magnitude of differences difficult to accurately quantify. In addition, these are selected groups of patients with HER2-positive (Table 5) and ER-positive (Table 6) early-stage disease enrolled in phenotype-specific clinical trials and, consequently, with an observed frequency of ER-positive disease that is lower and higher, respectively in the two groups, than that expected in the average newly diagnosed patient with breast cancer. The ER false-negative rate (central versus local testing) was in the range of 10%, whereas the false-positive rate was as high as 5% for ER (Table 5) and was much higher (> 10%) for PgR, most likely because of the low specificity of a recently available rabbit monoclonal antibody to PgR, SP2.[63]

Table 4. Concordance Between Primary Institution and Central Testing for ER in ECOG 2197

ER Testing at Primary Institution (IHC, institutional criteria for "positive")	ER Central Testing (IHC, Allred score ≥ 3) Positive No.	Positive %	Negative No.	Negative %	Total No.	Overall Concordance (%)* Rate	Overall 95% CI	Positive Concordance (%)† Rate	Positive 95% CI	Negative Concordance (%)‡ Rate	Negative 95% CI
Positive	382	89	27	8	409	90.2	87.9 to 92.3	88.8	85.5 to 91.7	92.0	88.6 to 94.7
Negative	48	11	312	92	360						
Total No.	430		339		769						

NOTE. Data adapted.[60]
Abbreviations: ER, estrogen receptor; ECOG, Eastern Cooperative Oncology Group; IHC, immunohistochemistry.

* Overall concordance was determined using the following formula: (total No. of results that were positive in both testing locations + total No. of results that were negative in both testing locations)/total No. of results for both tests.

† Positive concordance was determined using the following formula: total No. of results that were positive in both testing locations/total No. of positive results in central testing.

‡ Negative concordance was determined using the following formula: total No. of results that were negative in both testing locations/total No. of negative results in central testing.

Table 5. Central Pathology Review of ER in the Adjuvant Lapatinib and/or Trastuzumab Optimization Trial in Patients With HER2-Positive Breast Cancer

	Central ER Review (European Institute of Oncology)												
Local Laboratory Review	Positive (≥ 10% positive cells)		Positive (1% to 9% positive cells)		Negative		Total No. (N = 4,931)*	Overall Concordance (%)†		Positive Concordance (%)‡		Negative Concordance (%)§	
	No.	%	No.	%	No.	%		Rate	95% CI	Rate	95% CI	Rate	95% CI
Positive	2,481		54		113	4.3	2,648	87.7	86.7 to 88.6	83.7	82.3 to 84.9	94.1	92.9 to 95.0
Negative	388	16.9	107	4.7	1,788		2,283						

NOTE. Data adapted.[62]

Abbreviations: ER, estrogen receptor; HER2, human epidermal growth factor receptor 2.

* Seventy-nine percent of specimens submitted were confirmed centrally as HER2 positive, a subgroup of patients with a lower percentage of ER-positive tumors (approximately 50%) when compared with all newly diagnosed breast cancers.

† Overall concordance was determined using the following formula: (total No. of results that were positive [both ≥ 10 and 1% to 9% positive cells] in both testing locations + total No. of results that were negative in both testing locations)/total No. of results for both tests.

‡ Positive concordance was determined using the following formula: total No. of results that were positive [both ≥ 10 and 1% to 9% positive cells] in both testing locations/total No. of positive results [both ≥ 10 and 1% to 9% positive cells] in central testing.

§ Negative concordance was determined using the following formula: total No. of results that were negative in both testing locations/total No. of negative results in central testing.

GUIDELINE QUESTIONS

The overall purpose of this guideline is to improve the accuracy of hormone receptor testing and the utility of ER and PgR as prognostic and predictive markers for assessing in situ and invasive breast carcinomas. Therefore, this guideline addresses two principal questions regarding ER and PgR testing. Findings are listed in Table 7.

1. What is the optimal testing algorithm for determining ER and PgR status?
   1.1. What are the clinically validated methods that can be used in this assessment?

2. What strategies can ensure optimal performance, interpretation, and reporting of established assays?
   2.1. What are the preanalytic, analytic, and postanalytic variables that must be controlled to ensure that assay results reflect tumor ER and PgR status?
   2.2. What is the optimal internal quality management regimen to ensure ongoing accuracy of ER and PgR testing?
   2.3. What is the regulatory framework that permits application of external controls such as proficiency testing and on-site inspection?
   2.4. How can internal and external control efforts be implemented and their effects measured?

The Panel also reviewed a few special questions.

1. Should IHC of ER/PgR be performed in ductal carcinoma in situ (DCIS) or recurrent breast cancer specimens?

Table 6. Central Pathology Review of ER/PgR in the Adjuvant BIG 1–98 Trial Comparing Letrozole and Tamoxifen Given in Sequence or Alone in Patients With ER- and/or PgR-Positive Breast Cancer

	Central Review (European Institute of Oncology)												
Local Laboratory Review	Positive (≥ 10% positive cells)		Positive (1% to 9% positive cells)		Negative		Total No.	Overall Concordance (%)†		Positive Concordance (%)‡		Negative Concordance (%)§	
	No.	%	No.	%	No.	%		Rate	95% CI	Rate	95% CI	Rate	95% CI
ER							6,205*	97.6	97.2 to 98.0	98.7	98.4 to 98.9	26.7	17.9 to 37.0
Positive	5,980		54		66	1	6,100						
Negative	73	69.5	8	7.6	24		105						
PgR							5,237	80.2	79.1 to 81.3	81.8	80.7 to 82.9	33.0	29.1 to 37.1
Positive	3,584		247		183	4.6	4,014						
Negative	544	45.5	308	25.2	371		1,223						

NOTE. Data adapted.[61]

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor; BIG, Breast International Group.

* BIG 1–98 was an adjuvant endocrine therapy trial where 98% and 89% of tumors submitted were centrally confirmed as ER positive and PgR positive, respectively, a much higher percentage of hormone receptor–positive tumors when compared with all newly diagnosed breast cancers.

† Overall concordance was determined using the following formula: (total No. of results that were positive [both ≥ 10 and 1% to 9% positive cells] in both testing locations + total No. of results that were negative in both testing locations)/total No. of results for both tests.

‡ Positive concordance was determined using the following formula: total No. of results that were positive [both ≥ 10 and 1% to 9% positive cells] in both testing locations/total No. of positive results [both ≥ 10 and 1% to 9% positive cells] in central testing.

§ Negative concordance was determined using the following formula: total No. of results that were negative in both testing locations/total No. of negative results in central testing.

Table 7. Summary of Guideline Recommendations for ER and PgR Testing by IHC in Breast Cancer Patients

	Recommendation	Comments
Optimal algorithm for ER/PgR testing	Positive for ER or PgR if finding of $\geq$ 1% of tumor cell nuclei are immunoreactive. Negative for ER or PgR if finding of $<$ 1% of tumor cell nuclei are immunoreactive in the presence of evidence that the sample can express ER or PgR (positive intrinsic controls are seen). Uninterpretable for ER or PgR if finding that no tumor nuclei are immunoreactive and that internal epithelial elements present in the sample or separately submitted from the same sample lack any nuclear staining.	These definitions depend on laboratory documentation of the following: 1. Proof of initial validation in which positive ER or PgR categories are 90% concordant and negative ER or PgR categories are 95% concordant with a clinically validated ER or PgR assay.[3] 2. Ongoing internal QA procedures, including use of external controls of variable ER and PgR activity with each run of assay, regular assay reassessment, and competency assessment of technicians and pathologists. 3. Participation in external proficiency testing according to the proficiency testing program guidelines. 4. Biennial accreditation by valid accrediting agency.
Optimal testing conditions	Large, preferably multiple core biopsies of tumor are preferred for testing if they are representative of the tumor (grade and type) at resection.	Specimen should be rejected and testing repeated on a separate sample if any of the following conditions exist: 1. External controls are not as expected (scores recorded daily show variation). 2. Artifacts involve most of sample. Specimen may also be rejected and testing repeated on another sample if: 1. Slide has no staining of included normal epithelial elements and/or normal positive control on same slide. 2. Specimen has been decalcified using strong acids. 3. Specimen shows an ER-negative/PgR-positive phenotype (to rule out a false-negative ER assay or a false-positive PgR assay). 4. Sample has prolonged cold ischemia time or fixation duration $<$ 6 hours or $>$ 72 hours and is negative on testing in the absence of internal control elements.
	Interpretation follows guideline recommendation.	Positive ER or PgR requires that $\geq$ 1% of tumor cells are immunoreactive. Both average intensity and extent of staining are reported. Image analysis is a desirable method of quantifying percentage of tumor cells that are immunoreactive. H score, Allred score, or quick score may be provided. Negative ER or PgR requires $<$ 1% of tumor cells with ER or PgR staining. Interpreters have method to maintain consistency and competency documented regularly.
	Accession slip and report must include guideline-detailed elements.	
Optimal tissue handling requirements	Time from tissue acquisition to fixation should be as short as possible. Samples for ER and PgR testing are fixed in 10% NBF for 6 to 72 hours. Samples should be sliced at 5-mm intervals after appropriate gross inspection and margins designation and placed in sufficient volume of NBF to allow adequate tissue penetration. If tumor comes from remote location, it should be bisected through the tumor on removal and sent to the laboratory immersed in a sufficient volume of NBF. Cold ischemia time, fixative type, and time the sample was placed in NBF must be recorded. As in the ASCO/CAP HER2 guideline, storage of slides for more than 6 weeks before analysis is not recommended. Time tissue is removed from patient, time tissue is placed in fixative, duration of fixation, and fixative type must be recorded and noted on accession slip or in report.	

Continued		
	Recommendation	Comments
Optimal internal validation procedure	Validation of any test must be done before test is offered. See separate article on testing validation (Fitzgibbons et al[64]). Validation must be done using a clinically validated ER or PgR test method. Revalidation should be done whenever there is a significant change to the test system, such as a change in the primary antibody clone or introduction of new antigen retrieval or detection systems.	
Optimal internal QA procedures	Initial test validation. See separate article on testing validation (Fitzgibbons et al[64]). Ongoing quality control and equipment maintenance. Initial and ongoing laboratory personnel training and competency assessment. Use of standardized operating procedures including routine use of external control materials with each batch of testing and routine evaluation of internal normal epithelial elements or the inclusion of normal breast sections on each tested slide, wherever possible. Regular, ongoing assay reassessment should be done at least semiannually (as described in Fitzgibbons et al[64]). Revalidation is needed whenever there is a significant change to the test system. Ongoing competency assessment and education of pathologists.	
Optimal external proficiency assessment	Mandatory participation in external proficiency testing program with at least two testing events (mailings) per year.	
	Satisfactory performance requires at least 90% correct responses on graded challenges for either test.	Unsatisfactory performance will require laboratory to respond according to accreditation agency program requirements.
Optimal laboratory accreditation	On-site inspection every other year with annual requirement for self-inspection.	Reviews laboratory validation, procedures, QA results and processes, and reports. Unsuccessful performance results in suspension of laboratory testing for ER or PgR.

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor; IHC, immunohistochemistry; QA, quality assurance; NBF, neutral buffered formalin; ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists; HER2, human epidermal growth factor receptor 2.

2. Does PgR expression in breast cancer correlate with or influence the choice of endocrine therapy?

PRACTICE GUIDELINES

ASCO/CAP's practice guidelines reflect expert consensus based on the best available evidence. They are intended to assist physicians and patients in clinical decision making and to identify questions and settings for further research. With the rapid flow of scientific information in oncology, new evidence may emerge between the time an updated guideline was submitted for publication and when it is read or appears in print. Guidelines are not continually updated and may not reflect the most recent evidence. Guidelines address only the topics specifically identified in the guideline and are not applicable to interventions, diseases, or stages of diseases not specifically identified. Furthermore, guidelines cannot account for individual variation among patients and cannot be considered inclusive of all proper methods of care or exclusive of other treatments. It is the responsibility of the treating physician or other health care provider, relying on independent experience and knowledge of the patient, to determine the best course of treatment for the patient. Accordingly, adherence to any guideline is voluntary, with the ultimate determination regarding its application to be made by the physician in light of each patient's individual circumstances and preferences. ASCO/CAP guidelines describe the use of procedures and therapies in clinical practice and cannot be assumed to apply to the use of interventions in the context of clinical trials. ASCO and CAP assume no responsibility for any injury or damage to persons or property arising out of or related to any use of ASCO/CAP's guidelines or for any errors or omissions.

METHODS

Panel Composition

The ASCO Clinical Practice Guidelines Committee (CPGC) and the CAP Council on Scientific Affairs (CSA) jointly convened an Expert Panel (hereafter referred to as the Panel) consisting of experts in clinical medicine and research relevant to hormone receptor testing, including medical oncology, pathology, epidemiology, statistics, and health services research. Academic and community practitioners, a patient representative, and experts from the US National Cancer Institute (NCI) and international organizations were also part of the Panel. Representatives

from the FDA and the US Centers for Medicare and Medicaid Services served as ex-officio members. The opinions of Panel members associated with official government agencies like the US National Cancer Institute represent their individual views and not necessarily those of the agency with which they are affiliated. The Panel members are listed in Table 8. Representatives of commercial laboratories and assay manufacturers (Table 9) were invited as guests to attend the open portion of the 2-day meeting held at ASCO headquarters in Alexandria, VA, in December 2008. The planning, deliberations, and manuscript drafting were led by a six-member steering committee composed of two ASCO representatives (Drs Hayes and Wolff), two CAP representatives (Drs Hammond and Schwartz), and two additional experts in testing and evaluation of ER (Drs Allred and Dowsett).

Literature Review and Analysis

ASCO/Cancer Care Ontario (CCO) Systematic Review.— ASCO and CAP commissioned a systematic review of the literature on hormone receptor testing published since 1990. That review conducted by ASCO and CCO is being published separately (manuscript in preparation) and served as the primary source of the evidence for this guideline. Articles were selected for inclusion in the systematic review if they met the following prospective criteria. Studies comparing IHC in paraffin-embedded female breast cancer sections with another assay and comparative studies whose objectives were to improve or validate the quality of IHC studies that linked test performance to clinical outcome were specifically sought. Systematic reviews, consensus statements, and practice guidelines from 1990 onward were included if they addressed hormone receptor testing in female breast cancer using IHC in paraffin-embedded sections or gene expression signatures for ER and PgR. A cutoff date of 1990 was chosen because this was the time that IHC began to come into common use. Additional details of the literature search strategy are provided in the Systematic Review (manuscript in preparation).

ASCO/CAP Expert Panel literature review and analysis.— The Panel reviewed all data from the systematic review, as well as additional studies obtained from personal files.

Consensus Development Based on Evidence

The entire Panel met in December 2008, and additional work on the guideline was completed through e-mail and teleconferences of the Panel. The purpose of the Panel meeting was to refine the questions addressed by the guideline, draft guideline recommendations, and distribute writing assignments. All members of the Panel participated in the preparation of the draft guideline document, which was then disseminated for review by the entire Panel. The guideline was submitted to *Journal of Clinical Oncology* and *Archives of Pathology & Laboratory Medicine* for peer review. Feedback from external reviewers was also solicited. The content of the guidelines and the manuscript were reviewed and approved by the ASCO HSC and Board of Directors and by the CAP CSA and Board of Governors before publication.

Guideline and Conflict of Interest

The Expert Panel was assembled in accordance with ASCO's Conflict of Interest Management Procedures for Clinical Practice Guidelines (''Procedures,'' summarized at www.asco.org/guidelinescoi). Members of the Panel completed ASCO's disclosure form, which requires disclosure of financial and other interests that are relevant to the subject matter of the guideline, including relationships with commercial entities that are reasonably likely to experience direct regulatory or commercial impact as the result of promulgation of the guideline. Categories for disclosure include employment relationships, consulting arrangements, stock ownership, honoraria, research funding, and expert testimony. In accordance with the Procedures, the majority of the members of the Panel did not disclose any of these types of relationships. Disclosure information for each member of the Panel is published adjunct to this guideline.

Revision Dates

At biannual intervals, the Panel Co-Chairs and two Panel members designated by the Co-Chairs will determine the need for revisions to the guidelines based on an examination of current literature. If necessary, the entire Panel will be reconvened to discuss potential changes. When appropriate, the Panel will recommend revised guidelines to the ASCO CPGC, the CAP CSA, the ASCO Board, and the CAP Board for review and approval.

Definition of Terms

See Appendix for definitions of terms used throughout this document.

Summary of Outcomes Assessed

The primary outcome of interest was the correlation between hormone receptor status, as tested by various assays and methods, and benefit from endocrine therapy, as measured by prolongation of disease-free, progression-free, or overall survival or, in selected instances, response rates. Other outcomes of interest included the positive and negative predictive values, accuracy, and correlation of assays used to determine hormone receptor status, including (but not necessarily limited to) specific assay performance, technique, standardization attempted, quality assurance, proficiency testing, and individual or institutional training. Finally, improvement in assay results based on any of these interventions was examined.

Literature Search

The ASCO/CCO systematic review identified 337 studies that met the inclusion criteria.

RECOMMENDATIONS

What Is the Optimal Testing Algorithm for the Assessment of ER and PgR Status?

Summary and recommendations.—The Panel reviewed the literature on ER and PgR testing and discussed its implications for patients diagnosed with breast cancer. The purpose of both tests is to help determine likelihood of patients responding to endocrine therapy. Therefore, the optimal threshold to define clinical benefit should be based on thresholds that are clinically validated against patient outcome in patients treated with endocrine therapy compared with those who were not.

Table 8. Panel Members	
Panel Member	**Institution**
M. Elizabeth H. Hammond, MD, FCAP, Co-Chair	Intermountain Healthcare, University of Utah School of Medicine, UT
Antonio C. Wolff, MD, FACP, Co-Chair	The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, MD
Daniel F. Hayes, MD, Co-Chair	University of Michigan Comprehensive Cancer Center, University of Michigan Health System, MI
D. Craig Allred, MD, FCAP, Steering Committee Member	Washington University School of Medicine, St Louis, MO
Mitch Dowsett, PhD, Steering Committee Member	Royal Marsden Hospital, United Kingdom
Sunil Badve, MD	Eastern Cooperative Oncology Group, Indiana University, IN
Robert L. Becker, MD, Ex-Officio	US Food and Drug Administration, Center for Devices and Radiological Health, Office of In Vitro Diagnostic Device Evaluation and Safety
Patrick L. Fitzgibbons, MD, FCAP	St. Jude Medical Center, CA
Glenn Francis, MBBS, FRCPA, MBA	Princess Alexandra Hospital, Australia
Neil S. Goldstein, MD, FCAP	Advanced Diagnostics Laboratory, MI
Malcolm Hayes, MD	University of British Columbia, Canada
David G. Hicks, MD, FCAP	University of Rochester, NY
Susan Lester, MD	Brigham and Women's Hospital, MA
Richard Love, MD	Ohio State University, OH
Lisa McShane, PhD	National Cancer Institute, Biometric Research Branch, Division of Cancer Treatment and Diagnosis, MD
Keith Miller, MD	UK NEQAS, United Kingdom
C. Kent Osborne, MD	Baylor College of Medicine, TX
Soonmyung Paik, MD	National Surgical Adjuvant Breast and Bowel Project, PA
Jane Perlmutter, PhD, Patient Representative	Gemini Group, MI
Anthony Rhodes, PhD	University of the West of England, Bristol, UK NEQAS
Hironobu Sasano, MD	Tohoku University School of Medicine, Japan
Jared N. Schwartz, MD, PhD, FCAP	Presbyterian Hospital, NC
Fred C.G.J. Sweep, PhD	Radboud University, Nijmegen, the Netherlands
Sheila Taube, PhD	ST Consulting, Glen Echo, MD
Emina Emilia Torlakovic, MD, PhD	Royal University Hospital, Saskatoon, Canada
Giuseppe Viale, MD, FRCPath	European Institute of Oncology, and University of Milan, Italy
Paul Valenstein, MD, FCAP	St. Joseph Mercy Hospital, Ann Arbor, MI
Daniel Visscher, MD	University of Michigan, Ann Arbor, MI
Thomas Wheeler, MD, FCAP	Baylor College of Medicine, TX
R. Bruce Williams, MD, FCAP	The Delta Pathology Group, Shreveport, LA
James L. Wittliff, MD, PhD	University of Louisville, KY
Judy Yost, MA, MT (ASCP), Ex Officio	CMS, Division of Laboratory Services (CLIA), MD

Abbreviations: UK NEQAS, United Kingdom National External Quality Assessment Service; CMS, Centers for Medicare and Medicaid Services; CLIA, Clinical Laboratory Improvement Act.

What Are the Clinically Validated Methods That Can Be Used in This Assessment?

The earliest and arguably the most convincing and comprehensive clinical validation studies were conducted many years ago using LBAs correlating receptor status with response to endocrine therapy in patients with metastatic disease.[14] On the basis of a substantial number of published studies using LBAs, receptor testing by LBAs was officially established as the standard of care in the published proceedings of a symposium held in Bethesda, MD, in 1974.[14]

For many reasons, IHC on formalin-fixed, paraffin-embedded samples essentially replaced LBAs for assessing ER and PgR in breast cancer in the late 1980s.[65,66] One of the most comprehensive IHC studies of ER was published by Harvey et al,[19] who directly compared the predictive abilities of LBA and IHC (using antibody 6F11) in a large cohort of patients with newly diagnosed primary breast cancer. The patients in this study received a variety of types of adjuvant therapy, including none, endocrine alone (primarily tamoxifen), chemotherapy alone, and endocrine plus chemotherapy. Receptor status was scored as the sum of proportion and average intensity scores of positive staining tumor cells (the so-called Allred score on a scale ranging from 0 to 8). On the basis of a cut point analysis correlating IHC scores with outcome in patients treated with adjuvant endocrine therapy alone, they found that patients with tumors that had Allred scores ≥ 3 (corresponding to as few as 1% to 10% weakly positive cells) had a substantially and statistically significantly better prognosis than patients with scores less than 3 (ie, < 1% positive cells). Furthermore, the predictive ability of IHC in this study was superior to LBA results previously performed in the same tumors, and ER status by either method was not significantly associated with outcome in untreated patients or patients treated with chemotherapy alone. The study by Harvey et al[19] was based on samples prepared in an unconventional manner (particulate frozen tissue left over from the LBA assay, which was concentrated by centrifugation, fixed in 10% neutral [phosphate] buffered formalin [NBF] for 8 to 12 hours, and processed to formalin-fixed, paraffin-embedded samples). Since then, however, it has been

Table 9. Invited Guests to Open Session December 2008 Panel Meeting	
Invited Guests	**Affiliation**
Steven Shak, MD	Genomic Health, Redwood City, CA
Kenneth J. Bloom, MD	Clarient, Aliso Viejo, CA
Patrick Roche, PhD	Ventana Medical Systems, Tuscon, AZ
Allen M. Gown, MD	PhenoPath Laboratories, Seattle, WA
David L. Rimm, MD, PhD	Yale University, New Haven, CT
Hadi Yaziji, MD	Ancillary Pathways, Miami, FL
Richard Bender, MD	Agendia, Huntington Beach, CA
Roseanne Welcher	Dako, Glostrup, Denmark

Table 10. Well-Validated Assays for Evaluating Estrogen Receptor and Progesterone Receptor in Breast Cancer by Immunohistochemistry

Reference	Primary Antibody	Cut Point for ''Positive''
Estrogen receptor		
Harvey et al, 1999[19]	6F11	Allred score ≥ 3 (1% to 10% weakly positive cells)
Regan et al, 2006[22]; Viale et al, 2007[61]; Viale et al, 2008[72]	1D5	1% to 9% (low) and ≥ 10% (high)
Cheang et al, 2006[73]	SP1	≥ 1%
Phillips et al, 2007[74]	ER.2.123 + 1D5 (cocktail)	Allred score ≥ 3 (1% to 10% weakly positive cells)
Dowsett et al, 2008[48]	6F11	H score > 1 (≥ 1%)
Progesterone receptor		
Mohsin et al, 2004[70]	1294	Allred score ≥ 3 (1% to 10% weakly positive cells)
Regan et al, 2006[22]; Viale et al, 2007[61]; Viale et al, 2008[72]	1A6	1% to 9% (low) and ≥ 10% (high)
Phillips et al, 2007[74]	1294	Allred score ≥ 3 (1% to 10% weakly positive cells)
Dowsett et al, 2008[48]	312	≥ 10%

validated in other studies based on conventionally prepared samples.[20,59,67–69] For the most part, though, these studies represent analyses of prognosis in early-stage patients uniformly treated with tamoxifen (with the assumption that patients who fared less well did not respond to it) or are based on evidence of clinical response in the metastatic setting. Few directly address the predictive effect of ER for benefit from endocrine therapy compared with no therapy within prospectively conducted trials (Table 1). The panel considered this evidence and concluded that the harm from no treatment of patients with ER- and/or PgR-positive breast cancers was greater than the risk of overtreatment of some patients. Further evidence gathered after guideline implementation may potentially validate these assumptions. The same group of investigators published an analogous study that clinically validated PgR testing by IHC (based on antibody 1294).[70] Their assay for PgR has also received additional validation in more recent studies.[59,67]

Laboratory concordance with standards.—In the case of IHC assays of ER and PgR assays, there is no gold standard assay available. The Panel agreed that a relevant standard would be any assay whose specific preanalytic and analytic components conformed exactly to assays whose results had been validated against clinical benefit from endocrine therapy (clinical validation). Currently, there are several assay formats that meet this criterion as models against which a laboratory can compare its testing. Examples include the ER and PgR methods described in the publications by Harvey et al[19] and Mohsin et al[70] and the FDA 510(k)-cleared ER/PR pharmDx assay kit (Dako, Glostrup, Denmark). ER can also be determined by evaluation of RNA message, either by individual assay or as part of a multigene expression assay, such as a multigene array or as a multigene quantitative polymerase chain reaction. For example, the 21-gene recurrence score (RS) assay includes ER and PgR as one of the genes in the signature.[71] However, comparison between measures of ER/PgR protein by local IHC and of mRNA by central reverse transcription polymerase chain reaction showed a discordance rate of 9% and 12%, respectively,[60] and there are no published correlations of the individual measures of ER and PgR mRNA from the 21-gene signature with clinical outcome. As a result of this lack of published data correlating the ER and PgR individual measures within the 21-gene RS directly with clinical outcome, the committee concluded it was premature to recommend these individual measures for assay standardization and validation.

As discussed later, a laboratory performing ER testing should initially validate its proposed or existing assay against one of these clinically validated assays and demonstrate acceptable concordance. Details of acceptable validation methods are described in a separate publication.[64] To be considered acceptable, the results of the assay must be initially 90% concordant with those of the clinically validated assay for the ER- and PgR-positive category and 95% concordant for the ER- or PgR-negative category. Table 10 lists details of clinically validated assays including reagents, thresholds, and publications.

Definition of positive and negative ER and PgR tests.—The Panel deliberated carefully about recommending a universal cut point to distinguish ''positive'' and ''negative'' ER levels by IHC. The original cut point established for the LBAs in the 1970s was based primarily on the odds of response in the metastatic setting to a variety of endocrine treatments being used at the time in many centers.[14] Cytosol protein 10 fmol/mg was generally accepted as the optimum clinically useful cut point, and the FDA-approved kits using radiolabeled LBAs specified this value. Even then, the odds of responding for patients with ER levels less than 10 fmol/mg tissue were greater than 0, and others suggested that lower levels, such as more than 3 fmol/mg, might be appropriate.[75,76]

When IHC assays replaced LBAs in the early to mid-1990s, relatively few clinical studies were performed to establish optimum cut points for these assays. Instead, most studies simply compared the two and assumed that the IHC level corresponding to the previously determined LBA cut point was also valid. However, some early studies demonstrated that IHC was equivalent or superior to LBA in predicting benefit from adjuvant endocrine therapy.[19,70] Others showed significant correlations between ER levels determined by IHC and clinical outcome in patients with less advanced disease treated with adjuvant hormonal therapy (Tables 1 and 10).

IHC is equivalent or superior to LBA in predicting response to hormonal therapy, and IHC testing results as low as 1% positive-staining carcinoma cells are associated with significant clinical response (Tables 1 and 10). Given the substantial impact of tamoxifen and other endocrine therapies on mortality reduction and their relatively low toxicity profile, the Panel recommended that the cutoff to distinguish ''positive'' from ''negative'' cases should be ≥

1% ER-positive tumor cells. The Panel recommended considering endocrine therapy in patients whose breast tumors show at least 1% ER-positive cells and withholding endocrine therapy if less than 1%. We recognize that these recommendations will result in a slight increase in the application of endocrine therapy in some practices. We also recognize that it is reasonable for oncologists to discuss the pros and cons of endocrine therapy with patients whose tumors contain low levels of ER by IHC (1% to 10% weakly positive cells) and to make an informed decision based on the balance.

The percentage of stained tumor cells may provide valuable predictive and prognostic information to inform treatment strategies. Eight studies described the relationship between hormone receptor levels and patient outcomes.[18,20,48,77–81] Overall survival,[20,79,80] disease-free survival,[80] recurrence/relapse-free survival,[78,79] 5-year survival,[77] time to treatment failure,[20] response to endocrine therapy,[20,81] and time to recurrence[48] were all positively associated with ER levels. Overall survival,[20] time to treatment failure/progression,[18,20] response to endocrine therapy,[20,81] and time to recurrence[48] were positively related to PgR levels. These studies suggest that patients with higher hormone receptor levels will have a higher probability of positive outcomes and may influence oncologists' and patients' treatment decisions.

Although some studies suggest that the predictive role of PgR may not be as important clinically as ER,[18,61,82] other studies have shown that PgR status provides additional predictive value[70] independent of ER values,[81,83] especially among premenopausal women.[22,78] Again, predictive validity for PgR has been demonstrated with as few as 1% of stained tumor nuclei cells in retrospective studies.[70,81] Among patients who received adjuvant endocrine therapy, the best cutoff for both disease-free (adjusted $P = .0021$) and overall (adjusted $P = .0014$) survival was a total PgR Allred score of greater than 2, which corresponds to greater than 1% of carcinoma cells exhibiting weakly positive staining.[70] For patients with metastatic breast cancer who received first-line endocrine therapy on relapse, a correlation was found between PgR receptor status and response to endocrine therapy at a 1% staining threshold ($P = .044$) or response to tamoxifen therapy at 10% ($P = .021$) and 1% staining thresholds ($P = .047$). Furthermore, patients with carcinomas exhibiting $\geq$ 1% PgR staining levels had better survival after relapse ($P = .0008$).[81]

Reporting Results

Taking these issues into consideration, the Panel recommends that ER and PgR results be reported with three required result elements and two optional result elements (Table 7). The three required elements are as follows.

1. The percentage/proportion of tumor cells staining positively should be recorded and reported; all tumor containing areas of the tissue section on the slide should be evaluated to arrive at this percentage. The percentage can be arrived at either by estimation or by quantification, either manually by counting cells or by image analysis. Image analysis holds promise for improving inter- and intraobserver reproducibility, but controversy exists about how imaging should be implemented at this time. Standards of system performance have not yet been developed. If the sample is a cytology specimen, at least 100 cells should be counted or used to estimate the percentage of hormone receptor–positive tumor cells, particularly if the tumor specimen is limited and if the positive staining seems to involve only a minority of tumor cells.

2. The intensity of staining should be recorded and reported as weak, moderate, or strong; this measurement should represent an estimate of the average staining of the intensity of the positively stained tumor cells on the entire tissue section relative to the intensity of positive controls run with the same batch. Intensity is provided as a measure of assay quality over time and also allows for optional composite scoring.

3. An interpretation of the assay should be provided, using one of three mutually exclusive interpretations. The reader should provide an interpretation of the assay based on the following criteria.

- Receptor positive (either ER or PgR). The Panel recommends a cutoff of a minimum of 1% of tumor cells positive for ER/PgR for a specimen to be considered positive. There is no agreement about a range for receptor equivocal, so this term should not be used.
- Receptor negative. Tumors exhibiting less than 1% of tumor cells staining for ER or PgR of any intensity should be considered negative based on data that such patients do not receive meaningful benefit from endocrine therapy. The sample should only be considered negative in the presence of appropriately stained extrinsic and intrinsic controls. Any specimen lacking intrinsic elements (normal breast epithelium) that is negative on ER and/or PgR assay should be repeated using another tumor block or another tumor specimen and reported as uninterpretable rather than as negative.
- Receptor uninterpretable. The Panel agreed that there are no absolute assay exclusions. Nevertheless, a result should be considered uninterpretable if a sample did not conform to preanalytic specifications of the guideline, was processed using procedures that did not conform to guideline specifications or the laboratory's standard operating procedure, or the assay used to analyze the specimen was not validated and controlled as specified in the guideline. Examples of circumstances that may lead to uninterpretable results include testing of needle biopsies or cytology samples fixed in alcohol, use of fixatives other than 10% NBF (unless that fixative has been validated by the laboratory before offering the assay), biopsies fixed for intervals shorter than 6 hours or longer than 72 hours, samples where fixation was delayed for more than 1 hour, samples with prior decalcification using strong acids, and samples with inappropriate staining of internal assay controls (including intrinsic normal epithelial elements) or extrinsic assay controls. These conditions are not absolute because they depend on which conditions have been validated by the laboratory and which are subject to the judgment of the circumstances by the pathologist. The reason for an uninterpretable result should be specified (eg, fixation for $<$ 6 hours), and an alternative potential sample for retesting should be suggested, if appropriate.

Two optional report elements are recommended by the Panel, but not required.

1. A cautionary statement may be added to negative ER and PgR interpretations when the histopathology of the tumor is almost always associated with ER-positive and PgR-positive results. These include tubular, lobular, and mucinous histologic types or tumors with a Nottingham score of 1. The cautionary statement should indicate that although the patient's tumor tested as ER negative, tumors with the same histologic type or Nottingham score almost always test positive.

2. Using the percentage and intensity measurements provided, the pathologist may also provide a composite score such as the H score, Allred score, or quick score (Table 10). Because each of these is somewhat differently calculated and may lead to confusion across institutions, scoring is not required.

Appropriate populations to be tested.—The Panel developed consensus that ER and PgR status should be determined on all newly diagnosed invasive breast cancers. For patients with multiple synchronous tumors, testing should be performed on at least one of the tumors, preferably the largest. The Panel acknowledges that all newly diagnosed DCISs are also commonly being tested for ER and PgR. This practice is based on the results of a retrospective subset analysis of the NSABP B-24 clinical trial comparing tamoxifen versus placebo after lumpectomy and radiation, which has thus far been reported only in abstract form. There was a significant 40% to 50% reduction in subsequent breast cancer (ipsilateral and contralateral) restricted to patients with ER-positive DCIS at 10 years of follow-up, and a full manuscript has recently been submitted for peer review (personal communication from NSABP, September 2009). Because the results are scientifically reasonable and consistent with previous studies of invasive/metastatic breast cancer, the Panel sees value in assessing ER in patients with DCIS. However, because there are unlikely to be any validation studies, the Panel leaves it up to patients and their physicians to decide on testing, rather than making a formal recommendation. Breast recurrences should also always be tested to ensure that prior negative results of ER and/or PgR were not falsely negative and to evaluate the specimen for biologic changes since the previous testing.

What Strategies Can Ensure Optimal Performance, Interpretation, and Reporting of Established Assays?

Summary and recommendations.—The Panel considered those strategies that would ensure optimal performance of ER/PgR testing, interpretation, and reporting and was heavily influenced by the previous experience with the implementation of the elements included in the ASCO/CAP HER2 testing guideline. This guideline included measures to improve standardization of preanalytical variables, type of fixative and duration of tissue fixation, antibodies and controls, and assay interpretation.

2.1. What Are the Preanalytic, Analytic, and Postanalytic Variables That Must Be Controlled to Ensure That the Assays Reflect the Tumor ER and PgR Status?

Preanalytic standardization: tissue handling.—The warm and cold ischemic times are widely accepted as important variables in the analysis of labile macromolecules such as proteins, RNA, and DNA from clinical tissue samples. Warm ischemia time is the time from the interruption of the blood supply to the tumor by the surgeon to the excision of the tissue specimen; cold ischemia time is the time from excision to the initiation of tissue fixation. Numerous studies have documented the progressive loss of activity of these labile molecules after the surgical interruption of blood flow, leading to tissue ischemia, acidosis, and enzymatic degradation.[84–88] The contribution to this macromolecular degradation by the warm ischemic interval is currently under study. The standardization of the time between tissue removal and the initiation of fixation is an important step to help ensure that differences in levels of protein expression for clinically relevant targets such as ER are biologically meaningful and are not an artifact related to the manner in which the tissue was handled.

The breast resection specimen should be fixed as quickly as possible in an adequate volume of fixative (optimally 10-fold greater than volume of the specimen). The time of tissue collection (defined as the time that the tissue is handed from the surgical field) and the time the tissue is placed in fixative both must be recorded on the tissue specimen requisition to document the time to fixation of the specimen. The pathologist should effectively communicate this priority to all members of the breast care management team so processes are put in place to make sure these times are routinely recorded. It is the responsibility of the surgeon and operating room staff or the radiologist and his/her staff obtaining the specimen to document the collection time, and it is the responsibility of the pathologist and laboratory staff to document the fixation start time. Every effort should be made to transport breast excision specimens with a documented or suspected cancer from the operating room to the pathology laboratory as soon as they are available for an immediate gross assessment. The time from tumor removal to fixation should be kept to ≤ 1 hour to comply with these recommendations.

On receipt in the pathology laboratory, these specimens should be oriented and carefully inked for surgical margin assessment and then carefully sectioned at 5-mm intervals and placed in 10% NBF. Gauze pads or paper towels should be placed in between tissue slices to assist with the penetration of formalin into all areas of the tissue sample if the specimen will be further sectioned and placed into tissue cassettes at a later time. If gross tumor is easily identifiable, a small portion of tumor and fibrous normal breast tissue can be included together in a cassette and placed immediately into fixative at the time of the initial gross evaluation. This will initiate good tissue fixation and also ensure that normal breast elements are available as an internal positive control that have been handled and fixed in a manner that is identical to the tumor tissue. In situations where excision specimens are obtained remotely from the grossing laboratory, the pathologists should work with personnel in the remote operating suites to ensure that the sample is bisected through the tumor and promptly placed in NBF before transport. The time to insertion of tumor sample into fixative and the time of removal of the tumor from the patient should be noted on the specimen requisition by the remote personnel. Although less optimal than immediate gross examination of the fresh sample by the pathologist, this process is preferable to storage of the sample in the refrigerator unfixed or in fixative without sectioning.

Preanalytic standardization: type of fixative.—Only 10% NBF should be used as the fixative for breast tissue

specimens. Higher or lower concentrations of NBF are not acceptable. This recommendation is based on published literature regarding the expected or characteristic immunoreactivity for ER in breast cancer, which has been accrued over many years and has been clinically validated with patient outcomes in numerous clinical trials.[87] In addition, FDA approval for assay kits analyzing ER and HER2 explicitly states that formalin fixation should be used and that the FDA approval for the kits is not applicable if an alternative fixative is used. If the laboratory uses a formalin alternative for fixation, the assay must be validated against NBF fixation, and the laboratory director assumes responsibility for the validity of these assay results.

Preanalytic standardization: duration of tissue fixation.—Breast tissue specimens must be fixed in 10% NBF for no less than 6 hours and for not more than 72 hours before processing. Formalin is aqueous, completely dissolved formaldehyde. Formalin penetrates tissue at a rate of approximately 1 mm/h, which is the reason why breast excision samples must be incised in a timely fashion to initiate formalin fixation throughout the tissue. Fixation does not begin until formaldehyde has penetrated into the tissue. However, permeation of tissue by formalin is not the same as the chemical reaction of fixation, which involves protein cross-linking by formaldehyde. Chemical fixation requires time, with the rate limiting step being the equilibrium between formaldehyde and methylene glycol in solution, which is time dependent and can be measured in hours (clock reaction). Although complete tissue fixation usually requires 24 hours, published studies have documented that a minimum of at least 6 to 8 hours of formalin fixation for breast samples is needed to obtain consistent IHC assay results for ER.[88] Underfixation of breast tissue may lead to false-negative ER results. Overfixation is likely to be less problematic than underfixation but potentially could also lead to false-negative results as a result of excessive protein cross-linking by formaldehyde. This would particularly be true if there was inadequate antigen retrieval used during the performance of the ER/PgR assays (Table 11). Usual antigen retrieval protocols are optimized for 24 hours of fixation time.[96]

There is a misconception that smaller biopsy samples will fix more quickly than larger resection specimens and therefore require less time in formalin. Although formalin penetrates more quickly into these smaller samples, tissue fixation is a chemical reaction that requires time. As a result, small biopsy specimens require the same amount of fixation time as larger resection samples. Similarly, larger resection specimens must be incised in a timely manner to ensure adequate penetration of formalin so that the chemical reaction of fixation can begin. A number of studies comparing ER and PgR results from IHC assays on needle core biopsies and resection specimens from the same patient have suggested that the needle core may be a better tissue sample for ER/PgR testing by IHC because these tissues are often placed in formalin in a more timely fashion, will infiltrate more quickly because of their size, and thus may be exposed to more uniform and consistent tissue fixation. If core samples are large and representative of the resection specimen, the Panel recommends that such samples preferably be used for ER and PgR analyses if they have been fixed a minimum of 6 hours in 10% NBF.

Analytic standardization: antibody selection for ER testing.—The selection of antibodies for ER and PgR IHC testing should be restricted to those reagents that have well-established specificity and sensitivity and have been clinically validated, demonstrating good correlation with patient outcomes in published reports. Alternatively, the results of laboratory-selected antibodies should be at least 90% concordant with those of the clinically validated assay for the ER- and PgR-positive category and 95% concordant with those for the ER- or PgR-negative category that have been correlated with clinical outcomes of endocrine treatment. The Panel determined that the antibodies for ER that have met these criteria are clones 1D5, 6F11, SP1, and 1D5+ER.2.123, whereas the antibodies for PgR include clones 1A6, 1294, and 312 (Table 10). There is a single FDA 510(k)-cleared ER/PgR kit. Published reports have demonstrated that each of these antibodies is equivalent or superior to LBAs in terms of correlation with outcome and/or benefit from endocrine therapy (Tables 1 and 10). Antibodies sold as research use only or investigational use only or developed by the testing facility may not be used in ER and PgR testing. Use of research use only, investigational use only, and laboratory-developed antibodies in an assay is not compliant with these guidelines.

Analytic standardization: control samples for ER and PgR IHC assays.—Positive and negative controls should be included with every ER and PgR IHC assay batch run. Batch controls are used to monitor assay performance over time and to detect a loss of sensitivity or assay analytic drift. Acceptable batch controls include cell lines with defined receptor content varying from high positive to negative and including at least one intermediate level of receptor content. Other acceptable external controls include endometrial tissue with known receptor content. On-slide external controls and internal normal epithelial elements should be used to help ensure that all reagents were dispensed onto the slide containing a test sample and that the assay is performing properly. The internal positive control must display a heterogeneous staining pattern of the luminal cells, with a mixture of a variable number of cells exhibiting weak, moderate, and intense immunoreactivity. If the assay only highlights a few cells among the normal breast epithelium with a homogeneous staining pattern, then the risk of a false-negative assessment of the tumor ER and/or PgR is higher as a result of an insufficient sensitivity of the reaction to detect the tumor cells with a weak to moderate immunoreactivity. The normal breast tissue also represents a useful built-in negative control of the staining because the myoepithelial cells and the stromal cells must invariably show a negative result. In some specimens, there are no internal control elements (normal breast epithelium); in this case, the pathologist must exercise judgment as to whether the assay can be interpreted based on the level of ER and/or PgR positivity of the tumor cells, the histologic type of the tumor, the fixation status of the tumor, and the status of external controls.

To ensure that there has not been analytic drift because of subtle differences in technique or dilution, controls with intermediate reactivity or controls covering a spectrum of expression should be scored and recorded daily (percent positive tumor cells and intensity of staining) using laboratory standard scoring system or image analysis. It

Table 11. Influence of Fixative Duration on IHC ER and PgR Results

Reference	Comparison	No. of Samples	Fixation	Kit/Antibody	Antigen Retrieval	Results/Conclusion
Scharl et al[89]	FFPE *v* snap frozen	170; 82 additional snap frozen samples	2 fresh, PgR-rich breast cancer biopsies were divided into 4: 1 was snap frozen, and 3 were fixed in 5% formalin at RT at 12, 24, and 48 hours; in each case, 2 trypsinized paraffin sections and, if available, 2 fixed cryostat sections were incubated for IHC	Mi 60-10 (mPR1)	FFPE: digested with trypsin	Formalin fixation for 48 hours resulted in PgR detection, but staining was less intense than cryostat sections
Cavaliere et al[90]	Frozen section (FS) *v* paraffin section (PS)	115	Subdivided into 47 fresh (5–10 minutes) and 68 refrigerated (1–4 hours) samples from nearby hospital; primary breast cancers and 1 recurrent sample; used Cell Analysis System (CAS) image analysis	Frozen section: ER H222 and PgR KD68; paraffin section: ER 1D5 and PR 1A6	Microwaved in citrate buffer	For ER, frozen section *v* fresh: 91.5%; frozen section *v* refrigerated: 77.9%; for PgR, frozen section *v* fresh: 93.6%, frozen section *v* refrigerated: 83.9%
Arber[91]	Effect of various lengths of fixation on ER and PgR IHC results	33	10% NBF; duration of 24 hours to up to 154 days (range of maximum fixation, 7–154 days), with mean and median times of maximum formalin fixation of 53 and 42 days, respectively	1D5 and PR1A6	HIER buffer (pH 5.5–5.7; Ventana, Tucson, AZ)	ER/PgR staining will continue to immunoreact with antigen retrieval for up to 57 days
Goldstein et al[88]	Study 1 (prospective): 3 *v* 6 *v* 8 *v* 10 *v* 12 hours *v* 1 *v* 2 *v* 7 days of fixation; study 2 (retrospective): needle core biopsy *v* resection specimens	Study 1: 24; study 2: 9; control group: 36	10% NBF; tissue temporarily stored in 100% cold ethanol	1D5	25 or 40 minutes with EDTA buffer	Mean Q scores significantly different for 3, 6, and 8 hours ($P < .001$); Q score plateau after 6 to 8 hours; mean fixation time for needle core biopsy and resection specimens with similar results was 6.3 hours ($P = .01$); minimum fixation time for reliable IHC ER results is 6–8 hours, regardless of type or size of specimen
Ibarra et al[92]	Antibody cocktail *v* each antibody in cocktail; antibody cocktail normally fixed *v* overfixed	56	Fixed at RT in 10% phosphate/saline-buffered formalin for 90 minutes or 1, 3, or 7 days	ER mAb cocktail: 1D5 and LH1	Microwaved in citrate buffer; time prolonged up to a minimum of 20 minutes for overfixed tissue	No. of ER-positive samples decreased by 20%, 26%, and 35% after 7 days *v* 90 minutes of fixation with the ER cocktail, ER-1D5, and ER-LH1, respectively; antigen retrieval could recover some of the loss (1D5, 87%; LH1, 81%; cocktail, 97%)

Continued						
Reference	Comparison	No. of Samples	Fixation	Kit/Antibody	Antigen Retrieval	Results/Conclusion
Jensen and Ladekari[93]	Different fixation times	25, 9 were allowed to fix at different times	10% formalin within 15 minutes; duration: 2–4, 4–24, 24–48, and 48–166 hours, randomly assigned	ER (Dako, Glostrup, Denmark)	Microwave oven	No correlation between storage intervals (2–4, 4–24, 24–48, or 48–166 hours) and mean percentage of ER
von Wasielewski et al[94]	Various fixation types and times	3 cases divided into 11 portions variously fixed and processed	Fixed in 10% formalin or ethanol for various durations	ER: 1D5 and 6F11; PR: 1A6 and polyclonal antiserum (PgR, Dako)	Heat-induced epitope retrieval (microwaved in citrate buffer) with and without TAT	Best results with 24-hour incubation for ER and PgR; fixation for up to 4 days had little influence on ER or PgR detection; poor results with freezing plus cryosectioning before fixation, especially for ER; worst results with 12-hour delay in onset of fixation for ER and PgR; 6 hours = 92% and 85.3% immunoreactive relative to 24 hours in NBF for ER and PgR, respectively
von Wasielewski et al[95]	Various fixation types and times using TMAs	6 cases of breast carcinoma received immediately after surgical resection and divided into 22 portions	Fixed in 10% formalin or ethanol for various durations	1D5	Microwave epitope retrieval with citrate buffer	For TMA, 24 hours in NBF had best results; up to 72 hours in NBF did not alter ER detection; worst results with simulation of cryosectioning before fixation or delayed onset of fixation, followed by ethanol fixation; 6 hours = 80% immunoreactive relative to 24 hours in NBF

Abbreviations: IHC, immunohistochemistry; ER, estrogen receptor; PgR, progesterone receptor; FFPE, formalin-fixed, paraffin-embedded; RT, room temperature; NBF, neutral buffered formalin; mAb, monoclonal antibody; TMAs, tissue microarrays; TAT, turnaround time.

is not appropriate to use a single strong positive control tissue to evaluate assay performance.

If an external or internal control does not produce the expected reaction, the result of patient testing must not be reported. Instead, the assay should be repeated with the standard reagents under the standard conditions until acceptable ER and/or PgR reactivity of control material is achieved. No patient material should be reported until controls react appropriately.

If the particular histologic type of breast cancer is unlikely to be ER negative (tubular, mucinous, or lobular morphology or Nottingham score of 1), the tumor should also be subjected to confirmatory testing, such as sending the same specimen to a reference laboratory for retesting or by repeating the assay on another block or on a separate breast cancer specimen.

Postanalytic standardization: interpretation of IHC assays for ER and PgR.—The interpretation of ER and PgR assays should include an evaluation of both the percentage of positive tumor cell nuclei and the intensity of the staining reaction. The level of expression of ERs in different breast tumors demonstrates a broad dynamic range that can vary by several hundred–fold. There is still no consensus about what level of expression constitutes the equivocal range for ER/PgR, and this terminology should not be used in the report. Table 12 lists interpretation guidelines.

Postanalytic standardization: reporting of ER and PgR by IHC.—The elements to be recorded on an accession slip or reported are listed in Tables 13 and 14. The staining of normal breast elements, if present within the specimen, should also be reported as an additional check on the IHC assay performance.

Postanalytical standardization: ER and PgR IHC assay internal quality control and validation.—A comprehensive quality control program for ER/PgR IHC analyses should include all aspects of the total test including periodic trend

analysis to help ensure an appropriate and expected number of ER-positive breast cancers in the patient population served by the laboratory. Table 14 lists specific suggestions; additional suggestions are provided in a separate publication.[64]

What Is the Regulatory Framework That Allows for Increased Scrutiny?

The Clinical Laboratory Improvement Act of 1998 (CLIA 88) provides stringent quality standards for highly complex tests, which include all predictive cancer factor assays. This legislation also requires application of external controls to assure compliance with CLIA standards. These external controls include required successful performance on external proficiency surveys (or alternative external assessment of assay accuracy) and on-site biennial inspection of laboratories performing highly complex tests with defined criteria and actions required when performance is deemed deficient. On-site inspections may be performed by the Centers for Medicare and Medicaid Services or its agents or by various deemed private accreditors, including CAP, The Joint Commission, and COLA (formerly known as Commission on Office Laboratory Accreditation).

The FDA regulates medical devices as a result of the 1976 Medical Devices Amendments Act. ER and PgR testing reagents and kits, which have potentially high impact on patient mortality and morbidity, have been the subject of several guidance documents and reports referencing FDA opinion on the subject.[86]

After review of the legislation and applicable regulations, the Panel agreed that the current regulatory framework provided sufficient justification for the guideline recommendations without modification, just as it had for the previously published ASCO/CAP HER2 guideline. Other countries such as Australia and New Zealand have similar requirements.

What Are the Optimal External Quality Assurance Methods to Ensure Ongoing Accuracy in ER/PgR Testing?

Summary and recommendations.—The guideline is based on regulatory requirements of CLIA 88, published studies, previous CAP experience,[49,50] experience of other groups,[87] and the Panel's consensus.

Currently, there are no regulatory requirements for proficiency testing of ER or PgR assays in the United States. CLIA regulations require alternative assessment schemes for ER and PgR as substitutes for mandated successful performance on external proficiency testing. However, proficiency testing can be used to meet the alternative assessment requirement if it is available. The current guideline will make successful performance in proficiency testing mandatory. There are mandatory requirements for successful proficiency testing performance in Australia and New Zealand, which have been in place since 2001.

The guidelines also require enhanced levels of scrutiny at the time of laboratory inspection beyond those required by CLIA. The Panel recommends that ER and PgR testing be performed in a CAP-accredited laboratory or in a laboratory that meets the additional accreditation requirements set out within this guideline.

External quality assurance (laboratory accreditation).—Beginning in 2010, the CAP Laboratory Accreditation Program will require that every CAP-accredited laboratory performing ER and/or PgR testing participate in a proficiency testing program directed to these analytes. Other Centers for Medicare and Medicaid Services–approved certifying or accrediting organizations that wish to evaluate laboratory compliance with this guideline must bring their accreditation programs in conformance with this and other requirements.

The CAP Laboratory Accreditation Program will monitor performance in the required proficiency testing. Performance less than 90% (described in detail in the following section) will be considered unsatisfactory and will require internal or external response consistent with accreditation program requirements. Responses must include identification of the cause of the poor performance, actions taken to correct the problem, and evidence that the problem has been corrected. Competency of the laboratory personnel performing the ER/PgR testing, including the pathologists, is an important aspect of the laboratory proficiency. Competency of testing personnel and pathologists must be assured by the laboratory director of each facility in a manner consistent with CLIA. Competency assessments must be documented, and documentation shall be evaluated at the time of laboratory inspection accreditation. The checklist of requirements for laboratories is presented in Table 15.

Proficiency testing requirements.—All laboratories reporting ER and/or PgR results must participate in a guideline-concordant proficiency testing program specific for each assay and method used. To be concordant with this guideline, proficiency testing programs must distribute specimens at least twice per year including a sufficient number of challenges (cases) to ensure adequate assessment of laboratory performance. For programs with ≥ 10 challenges per event, satisfactory performance requires correct identification of at least 90% of the graded challenges in each testing event. Laboratories with less than 90% correct responses on graded challenges in a given proficiency testing event are at risk for the next event. Laboratories that have unsatisfactory performance will be required to respond according to accreditation program requirements up to and including suspension of ER and/or PgR testing for the applicable method until performance issues are corrected. In some Canadian provinces and within the United Kingdom, the method of proficiency testing is different. In Canada, laboratories may participate in proficiency testing that uses sections of tissue microarrays offered by the Canadian Immunohistochemistry Quality Control (an academic program associated with the Canadian Association of Pathologists) or tumor samples or sections of cell blocks with characterized cell lines. Many Canadian laboratories also participate in CAP proficiency testing programs or European programs. The results may or may not be used for laboratory accreditation depending on the province. Laboratories receive unstained materials and must return those materials to a central laboratory for review and comment. The Australasian program developed by the Royal College of Pathologists of Australasia Quality Assurance Program consists of two components. Laboratories are sent unstained sections from tissue microarray blocks and are required to stain these and return them for central review and scoring. In addition, laboratories are required to submit de-identified data on the ER/PgR and

Table 12. IHC ER/PgR Testing Interpretation Criteria
Review controls (external standard and internal normal breast epithelium if present). If not as expected, the test should be repeated and not interpreted
Provide an interpretation of the assay as receptor positive, receptor negative, or receptor uninterpretable.
Positive interpretation requires at least 1% of tumor cells showing positive nuclear staining of any intensity.
Receptor negative is reported if < 1% of tumor cells show staining of any intensity.
Receptor uninterpretable is reported if the assay controls are not as expected or the preanalytic or analytic conditions do not conform to the guideline and there is no tumor cell staining in the absence of normally stained intrinsic epithelial elements.
Report the percentage of cells with nuclear staining using either estimation or quantitation. Quantitation may be done either by image analysis or manually.
Entire slide should be reviewed to assess the tumor-containing areas. Cytology samples with limited tumor cells and little tumor staining must have at least 100 cells counted.
Report an average intensity of tumor cell nuclei recorded as strong, moderate, or weak.
A score may be provided if the scoring system is specified.
Quantitative image analysis is encouraged for samples with low percentages of nuclear staining or in cases with multiple observers in the same institution. It is also a valuable way to quantify intensity and assure day-to-day consistency of control tissue reactivity.
If cytoplasmic staining occurs, repeat assay or perform on another sample.
Reject sample if normal ducts and lobules do not show obvious staining of some cells with variable intensity in the presence of totally negative tumor cells.
Reject sample if there are obscuring artifacts such as from decalcification of sample or staining only of necrotic debris.
In samples with DCIS only, the type of DCIS should be mentioned and the DCIS may be scored for ER/PgR; in patients with invasive disease and DCIS, ER/PgR should be reported only for the invasive component. DCIS staining pattern may also be provided in a comment
The ER and PgR results should fit the clinical profile of the patient being evaluated: Consider the type of invasive cancer and the grade of the cancer in interpretation; some cancer types like lobular, mucinous, and tubular carcinoma are almost always strongly ER positive and only rarely ER negative.

Abbreviations: IHC, immunohistochemistry; ER, estrogen receptor; PgR, progesterone receptor; DCIS, ductal carcinoma in situ.

HER2 status of reported breast cancers for evaluation of acceptable performance. Enrollment and participation in these programs are mandatory.

How Can These Efforts Be Implemented and the Effects Measured?

Plans to ensure compliance with guideline.—ASCO and CAP will provide educational opportunities (print, online, and society meetings) to educate health care professionals, patients, third-party payers, and regulatory agencies. In addition, CAP is producing a certificate program for pathologists that will assess their competency in following both the hormone receptor and the HER2 guideline recommendations. CAP will urge its members and participants in accreditation and proficiency testing programs to optionally append a statement to individual results or laboratory informational or promotional materials indicating that the laboratory's ER/PgR assays have been validated and performed in accordance with ASCO/CAP ER testing guidelines, provided that all of the guideline conditions are met.

ASCO and CAP will work to coordinate these recommendations with those of other organizations, such as the National Comprehensive Cancer Network, the Commission of Cancer of the American College of Surgeons, the American Joint Committee on Cancer, and patient advocacy organizations.

We are confident that these guidelines and measures developed for testing of ER, PgR, and HER2 will improve performance of laboratories using these and future predictive testing methods. CAP will actively review results of proficiency testing and laboratory accreditation activities and periodically publish performance results.

CAP will also work to include quality monitoring activities of ER and PgR testing in its programs designed for ongoing quality assessment, similar to its Q-tracks and Q-probes. In Australasia, participation in the programs is mandatory and linked to laboratory accreditation. In Australia and New Zealand, the laboratory accreditation is linked to funding of testing for laboratories ensuring compliance.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Employment or Leadership Position: Jared N. Schwartz, Aperio (C). **Consultant or Advisory Role:** Mitch Dowsett, Dako (C); D. Craig Allred, Genomic Health (C), Clarient (C), Dako (C); Sunil Badve, Dako (C); Neal S. Goldstein, Clarient (C); Giuseppe Viale, Dako (C). **Stock Ownership:** D. Craig Allred, Clarient. **Honoraria:** Glenn Francis, Roche Ventana Medical Systems; Giuseppe Viale, Dako. **Research Funding:** Hironobu Sasano, Ventana Japan. **Expert Testimony:** None. **Other Remuneration:** Glenn Francis, Roche Ventana Medical Systems.

After the guideline manuscript was completed, Jared N. Schwartz assumed an Employment or Leadership Position with Aperio and resigned as co-chair of the Expert Panel.

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Table 13. Elements to Be Included in Accession Slip for ER and PgR Assays
Patient identification information
Physician identification
Date of procedure
Clinical indication for biopsy
Specimen site and type of specimen
Collection time
Time sample placed in fixative
Type of fixative
Fixation duration

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor.

Table 14. Reporting Elements for ER and PgR IHC Assays

- Patient identification information*
- Physician identification*
- Date of service*
- Specimen site and type*
- Specimen identification (case and block number)*
- Fixative
- Cold ischemia time (time between removal and fixation)
- Duration of fixation
- Staining method used
  - Primary antibody and vendor
  - Assay details and other reagents/vendors
  - References supporting validation of assay (note: most commonly, these will be published studies performed by others that the testing laboratory is emulating)
- Status of FDA approval
- Controls (high protein expression, low-level protein expression, negative protein expression, internal elements or from normal breast tissue included with sample)
- Adequacy of sample for evaluation
- Results*
  - Percentage of invasive tumor cells exhibiting nuclear staining†
  - Intensity of staining: strong, medium, or weak
  - Interpretation:
    - Positive (for ER or PgR receptor protein expression), negative (for ER or PgR protein expression), or uninterpretable.
    - Internal and external controls (positive, negative, or not present)
    - Standard assay conditions met/not met (including cold ischemic time and fixation parameters)
    - Optional score and scoring system
    - Comment: Should explain reason for uninterpretable result and or any other unusual conditions, if applicable; may report on status of any DCIS staining in the sample; should also provide correlation with histologic type of the tumor; may provide information about laboratory accreditation status

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor; IHC, immunohistochemistry; FDA, US Food and Drug Administration.

* Report should contain these elements as a minimum. Other information must be available in the laboratory for review and/or appear on the patient accession slip.

† There is no recommendation in this guideline concerning whether specimens containing only ductal carcinoma in situ should be tested for ER/PgR.

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Table 15. CAP Laboratory Accreditation Elements Requiring Documentation

- Validation of test method before reporting patient results
- Use and following of standard operating procedures with appropriate elements and sign-offs
- Qualifications, responsibilities, and training of personnel involved in testing
- Proper labeling of samples and reagents
- Proper storage and handling of samples and reagents
- Equipment calibration, maintenance, QC, and remedial action; proficiency testing performance and corrective actions when 100% not achieved
- Internal QA plan for entire testing process, evidence that it is followed, and identified problems monitored and resolved effectively
- Quality of tests for interpretation
- Ongoing competency assessment of technologists and pathologists*
- Report adequacy and quality, including required dates and times
- Recordkeeping for entire test process and record retention
- Accurate, timely submission of results

Abbreviations: CAP, College of American Pathologists; QC, quality control; QA, quality assurance.

* Competency assessment is monitored by periodic or continuous review of performance of those doing tests against peers. When failure is documented, remediation is undertaken.

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APPENDIX

Definitions

Analyte-specific reagent.—Antibodies, both polyclonal and monoclonal, specific receptor proteins, ligands, nucleic acid sequences, and similar reagents, which, through specific binding or chemical reaction with substances in a specimen, are intended for use in a diagnostic application for identification and quantification of an individual chemical substance or ligand in biologic specimens [21CFR864.4020(a)].

Research use only (RUO).—Products that are in the laboratory research phase of development (ie, either basic research or the initial search for potential clinical utility) and not represented as an effective in vitro diagnostic product (21CFR809.10).

Investigational use only (IUO).—A product being shipped or delivered for product testing before full commercial marketing (for example, for use on specimens derived from humans to compare the usefulness of the product with other products or procedures that are in current use or recognized as useful) (21CFR809.10).

Clinical laboratory.—A facility for the biologic, microbiologic, serologic, chemical, immunohematologic, hematologic, biophysical, cytologic, pathologic, or other examination of materials derived from the human body for the purpose of providing information for the diagnosis, prevention, or treatment of any disease or impairment of, or the assessment of the health of, human beings. These examinations also include procedures to determine, measure, or otherwise describe the presence or absence of various substances or organisms in the body. Facilities only collecting or preparing specimens (or both) or only serving as a mailing service and not performing testing are not considered laboratories (42CFR493.2).

US Food and Drug Administration (FDA)–cleared test.—A test that has been cleared by the FDA after analysis of data showing substantial performance equivalence to other tests being marketed for the same purpose. Such tests typically follow the 510(k) approval route (21CFR807).

FDA-approved test.—A test that is classified as a class III medical device and that has been approved by the FDA through the premarket approval process (21CFR814.3).

Laboratory modified test.—An FDA-cleared or FDA-approved test that is modified by a clinical laboratory, but not to a degree that changes the stated purpose of the test, approved test population, specimen type, specimen handling, or claims related to interpretation of results.

Laboratory developed test (LDT).—A test developed within a clinical laboratory that has both of the following characteristics: is performed by the clinical laboratory in which the test was developed and is neither FDA cleared nor FDA approved.

Note: All laboratory modified tests are, by definition, LDTs. An LDT may or may not use analyte-specific reagent, RUO, or IUOs; the type of reagents and devices used does not affect whether a test is classified as an LDT. A laboratory is considered to have developed a test if the test procedure or implementation of the test was created by the laboratory performing the testing, irrespective of whether fundamental research underlying the test was developed elsewhere or reagents, equipment, or technology integral to the test was purchased, adopted, or licensed from another entity.

Validation of a test.—Confirmation through a defined process that a test performs as intended or claimed.

Note: There is no universally acceptable procedure for validating tests. The process for validating tests must take into account the purpose for which a test is intended to be used, claims made about the test, and the risks that may prevent the test from serving its intended purpose or meeting performance claims. Even FDA-approved and FDA-cleared tests require limited revalidation in clinical laboratories (a process often referred to as verification) to establish that local implementation of the test can reproduce a manufacturer's validated claims. Tests that use reagents or equipment that have not been validated (such as RUOs or IUOs) typically pose increased risks that require more extensive validation, as do tests used in more loosely controlled settings. The determination of whether a test has been adequately validated requires professional judgment.

Verification of a test.—An abbreviated process through which a clinical laboratory establishes that its implementation of an FDA-approved and FDA-cleared test performs in substantial conformance to a manufacturer's stated claims.

Analytic validity.—A test's ability to accurately and reliably measure the analyte (measurand) of interest. The elements of analytic validity include the following, as applicable.

- Accuracy. The closeness of agreement between the average value obtained from a large series of measurements and the true value of the analyte. Note: Technically, the term accuracy refers to the measure of the closeness of a single test result to the true value, not the average of multiple results. The definition of accuracy used here is what metrologists call trueness of measurement and describes the popular (but technically incorrect) meaning of the word accuracy.
- Precision. The closeness of agreement between independent results of measurements obtained under stipulated conditions (the International Organization of Standardization 1993).
- Reportable range. For quantitative tests, the span of test result values over which the laboratory can establish or verify the accuracy of the instrument or test system measurement response and over which results will be reported. For semiquantitative, binary, and nominal/categoric tests, the reportable range is all of the values that can be reported by the test system (eg, 2+, 3+, ''positive,'' ''negative,'' *Escherichia coli*, *Staphylococcus aureus*).
- Analytic sensitivity. For quantitative tests (including semiquantitative tests), analytic sensitivity is the lowest amount of analyte (measurand) in a sample that can be detected with (stated) probability, although perhaps not quantified as an exact value. For qualitative tests (binary and nominal/categoric tests), analytic sensitivity is the proportion of instances in which the analyte/measurand/identity is correctly detected, within a stated CI.
- Analytic specificity. Ability of a measurement procedure to measure solely the measurand/analyte.

Note: Analytic validity is expressed in the context of a defined set of test conditions (including standard operating procedures and permissible specimen types) and an ongoing quality management regimen (including, as applicable, ongoing quality control, periodic assay recalibration, and external proficiency testing or alternative external testing). If the test conditions or quality management regimen changes, the analytic validity of a test may change.

Clinical validity.—A test's ability to detect or predict a disorder, prognostic risk, or other condition or to assist in the management of patients. The elements of clinical validity include the following, as applicable.

- Clinical sensitivity (clinical detection rate). The proportion of individuals with a disorder, prognostic risk, or condition who are detected by the test.
- Clinical specificity. The proportion of individuals without a disorder, prognostic risk, or condition who are excluded by the test.
- Reference limits. A value or range of values for an analyte that assists in clinical decision making. Reference values are generally of two types—reference intervals and clinical decision limits. A reference interval is the range of test values expected for a designated population of individuals. This may be the central 95% interval of the distribution of values from individuals who are presumed to be healthy (or normal). For some analytes that reflect high-prevalence conditions (such as cholesterol), significantly less than 95% of the population may be healthy. In this case, the reference interval may be something other than the central 95% of values. A clinical decision limit represents the lower or upper limit of a test value at which a specific clinical diagnosis is indicated or specified course of action is recommended.
- Clinical utility. The clinical usefulness of the test. The clinical utility is the net balance of risks and benefits associated with using a test in a specific clinical setting. Clinical utility does not take into consideration the economic cost or economic benefit of testing and is to be distinguished from cost-benefit and cost-effectiveness analysis. Clinical utility focuses entirely on the probabilities and magnitude of clinical benefit and clinical harm that result from using a test in a particular clinical context.

Note 1: The qualities listed in this Appendix represent the primary performance measurements that are used to describe the clinical capabilities of a test. Other measures of clinical validity may be applicable in particular circumstances.

Note 2: Clinical validity is expressed in the context of a defined test population and a defined testing procedure. If the test population changes (eg, a change in the prevalence of disease) or the testing procedure changes, the clinical validity of a test may change.